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Method for Isolation and Identification of *Corynebacterium vaginale* (*Haemophilus vaginalis*)

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A method for the clinical isolation and recognition of *Corynebacterium vaginale* (*Haemophilus vaginalis*) is presented. Wet mount and stained characteristics of genital tract discharges, cellular and colonial morphology of the bacilli, inhibition by H_2O_2, lack of a catalase, and fermentation of particular carbohydrates are the determinant factors. The method enables differentiation of the species from unclassified diphtheroids common to the genitourinary tract.

Corynebacterium vaginale (*Haemophilus vaginalis*) is thought to be the major cause of a mild, venereally transmitted vaginitis (6). The bacterium has been reported to be associated with puerperal pyrexia (4) and a few cases of nongonococcal urethritis (3, 8, 11); it has been isolated from aseptically aspirated urine (10) and peripheral blood after septic abortion (15).

The organism was originally cultivated on blood-agar and classified as *Haemophilus vaginalis* (6, 8). Recent success in cultivating it in a medium containing only casein hydrolysate, carbohydrates, vitamins, nucleic acid bases, salts, and trace metals was conclusive evidence that this bacillus cannot be a legitimate member of genus *Haemophilus* (2). True *Haemophilus* species require hemin (X factor), nicotinamide adenine dinucleotide (V factor), or other definable coenzyme-like substances (16). Zinnemann and Turner (17) have recommended reclassification as *Corynebacterium vaginale* on the basis of morphology and staining properties.

Special methods are required for primary isolation and maintenance. Neither blood nor yeast extract is necessary for cultivation, but the extensive B-vitamin and purine-pyrimidine requirements of the bacillus must be met by the proteose-peptone utilized. Proteose Peptone No. 3 (Difco) is satisfactory as to the nitrogen, vitamin, and nucleic acid base requirements in a medium developed for this organism (2). Once isolated, *C. vaginale* must be differentiated from other *Corynebacterium* species and unclassified diphtheroid-like organisms common to the genitourinary tract.

A method is presented that has proved effective for isolation and identification of the bacterium from vaginal and urethral specimens even when plates are heavily populated with streptococci, yeasts, lactobacilli, unclassified diphtheroids, etc. All previously described methods have utilized blood, which precluded examination of colony forms by transmitted light, and called for serum in carbohydrate fermentation media. The carbohydrates and enzymes contained in serum make it difficult to obtain accurate information on bacterial activity in such media. The principal features of this new method are employment of a clear medium which allows observation of colony morphology by transmitted light and the determination of prime fermentation reactions without interference from blood or serum.

MATERIALS AND METHODS

Transport medium. Difco Proteose Peptone No. 3, 1.5% in distilled water, is satisfactory for transport of vaginal swabs to the laboratory. Adjust broth to *p*H 6.8 before autoclaving, and dispense 2 ml in screw-cap tubes (16 by 125 mm). Tighten caps to prevent evaporation during storage.

Isolation medium. Peptone-starch-dextrose (PSD) medium was developed by Dunkelberg and McVeigh (2) for cultivation of *C. vaginale*. It has the following formulation: Proteose Peptone No. 3, 2.0% (the original formula called for 1.5%); soluble starch, 1.0%; dextrose, 0.2%; Na_2HPO_4, 0.1%; $NaH_2PO_4 \cdot H_2O$, 0.1%; and agar, 1.5%. To make 1 liter, add starch to 100 ml of cold water and mix. Add cold water containing starch to 400 ml of boiling water and then add the remaining water (500 ml) and ingredients. Adjust *p*H to 6.8, autoclave for 15 min at

121 C, cool to approximately 55 C, and pour 20 to 25 ml per plate.

Maintenance medium. Maintenance medium was the same as isolation medium, except that only 0.8% agar was used. Pipette 6 to 10 ml into test tubes and store in the refrigerator. Before use, medium should be melted in a boiling-water bath and then slanted. Inoculate by making multiple stabs and streaking on the slant surface. This medium is also used to test for the presence of catalase.

Fermentation media. Fermentation medium contained Proteose Peptone No. 3, 2.0%; agar, 0.5%; bromocresol purple (1.6% dye in ethyl alcohol), 1.0 ml per liter; and carbohydrate, 1.0%. To make 100 ml of medium, place peptone, agar, and indicator solution in 90 ml of distilled water. Individual carbohydrate solutions are made by adding 10 g of sugar to distilled water of sufficient quantity to make 100 ml. Autoclave the peptone and sugar solutions separately for 15 min at 121 C, cool to approximately 55 C, and then mix 10 ml of sugar solution with 90 ml of the peptone-agar solution. Aseptically pipette 10 ml of complete medium into sterile test tubes with screw caps. Carbohydrate media required are dextrose, maltose, starch, and PSD without buffers but containing 0.5% agar and bromocresol purple indicator.

Stereoscopic microscope. A stereoscopic (dissection) microscope with substage light source and a clear glass stage is required for examination of colony morphology. The Spencer AO series 58M-2 instrument is satisfactory.

RESULTS

Figure 1 is a diagrammatic illustration of the protocol we use to recover *C. vaginale* from vaginal specimens. Vaginal swabs are removed from the transport medium [(1), Fig. 1], and one of them is used to prepare a smear for Gram staining. A wet mount is prepared from the transport tube contents [(2), Fig. 1]. The wet mount is

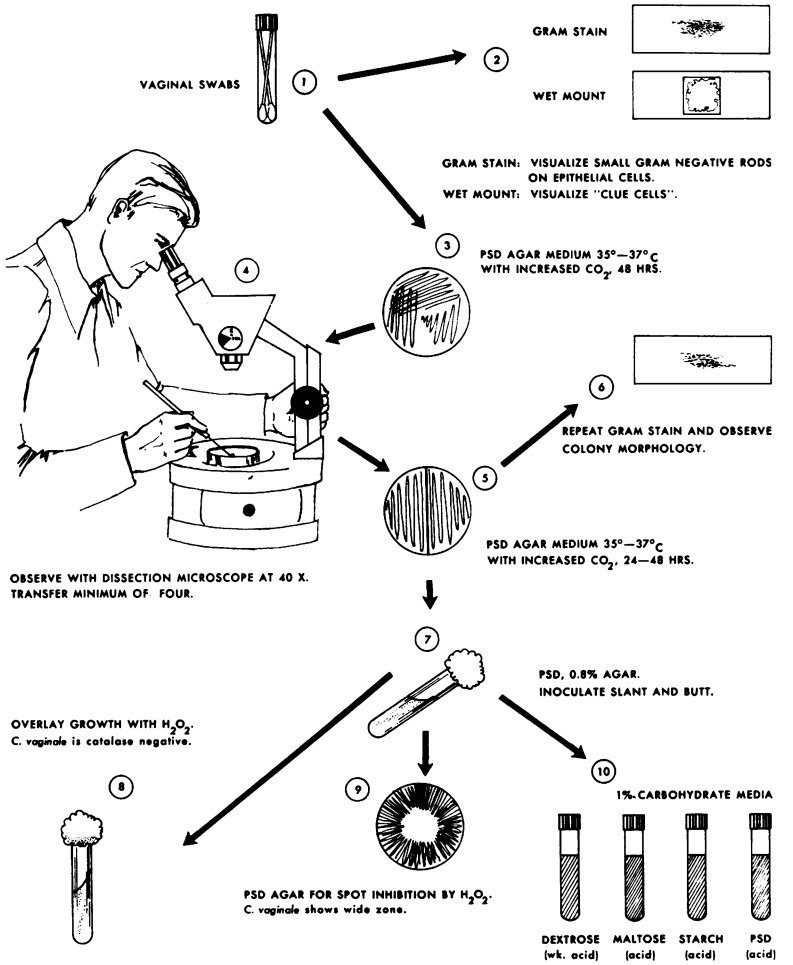

FIG. 1. *Diagrammatic outline of the protocol for isolation and identification of C. vaginale.*

examined with the high-dry objective for "clue cells" (6), whose presence is often significant (1). "Clue cells" are defined as squamous epithelial cells whose surfaces are covered by great numbers of the bacilli. These epithelial cells present a distinct granular appearance when viewed in wet mounts.

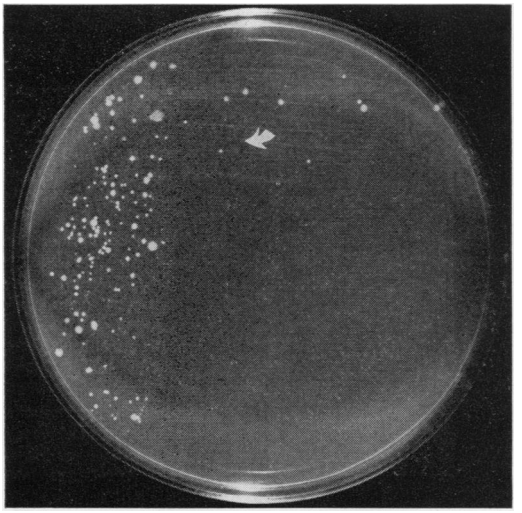

FIG. 2. *Vaginal swab streak-out on PSD agar after 48 hr of incubation. Small white colonies are C. vaginale. Larger gray colonies (arrow) inhibiting C. vaginale are streptococci. Large white colonies at top and left positions are mixed staphylococci, micrococci, etc.*

The other vaginal swab is touched to a PSD agar plate, and the material is streaked for isolation with a sterile loop [(3), Fig. 1]. Fine streaking is essential to ensure separate colonies. Plates are incubated for 48 hr at 35 to 37 C in candle extinction jars (Fig. 2).

After incubation, the plates are examined carefully for *C. vaginale* colonies [(4), Fig. 1], which are distinctive but not necessarily unique. In many cases, *C. vaginale* constitutes the predominant flora, and one has no difficulty finding these organisms. At other times, they are mixed with a variety of streptococci, micrococci, lactobacilli, yeast, and other species. The organisms most likely to cause confusion with *C. vaginale*, however, are other *Corynebacterium* species and unclassified diphtheroids. We use a stereoscopic microscope with a total magnification of 40× to examine colonies. *C. vaginale* appears to produce only one colony form, although the colony size is variable. This form is described as follows. On PSD agar after 48 hr of incubation, the colonies have a diameter of 0.5 to 2.0 mm, dull white color, convex, domed, somewhat conical shape, a dense or compact appearance, entire border with no lobations, and few, if any, irregularities. The

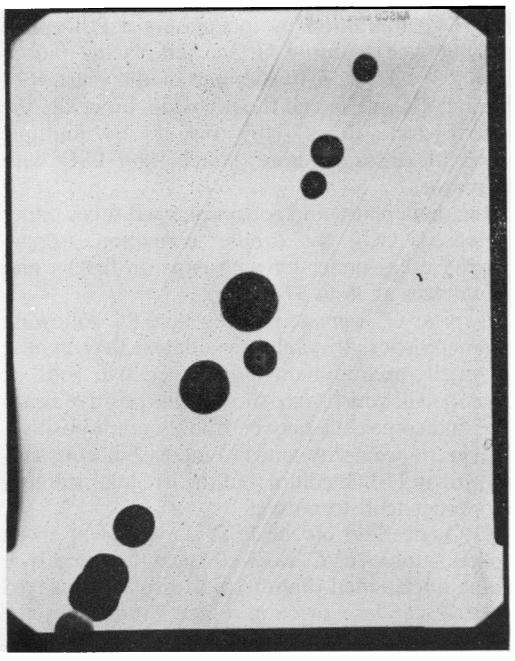

FIG. 3. *Colonies of a clinical isolate, C. vaginale, on PSD agar after 48 hr of incubation.* ×40.

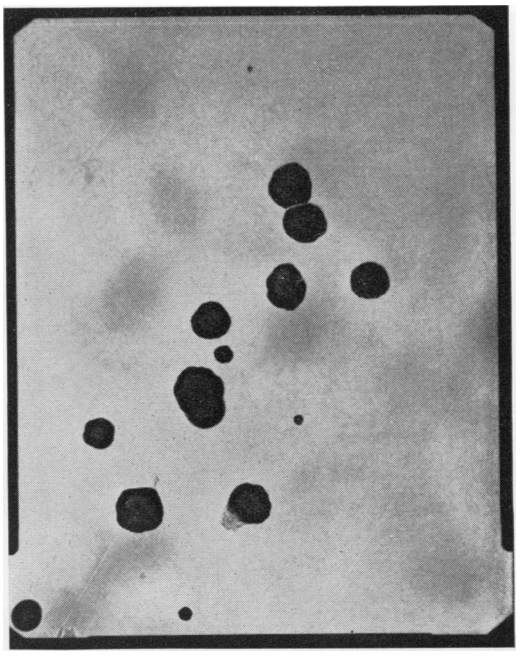

FIG. 4. *Colonies of an unclassified diphtheroid on PSD agar after 48 hr incubation.* ×40.

compact appearance is an optical effect caused by the coloration of the colony and does not imply actual density. Fresh clinical isolates tend to the smaller colony size and reference strains with a long laboratory history tend to the larger size, but the morphology remains uniform (Fig. 3).

After finding suspect colonies, reach under the objective of the stereoscopic microscope (there is a working distance of approximately 10 cm between the objective and the agar surface) with a fine, sterile loop and carefully pick up a colony while observing it through the microscope. Transfer at least four colonies to sectors on PSD plates [(5), Fig. 1]. This may appear difficult at first; but, with practice, it can be done with alacrity. Avoid picking two adjacent colonies in one movement because they seem to have identical morphology, as this may lead to mixed cultures. Figures 4 to 7 illustrate colonial morphology of some other species found on PSD agar.

Incubate the isolates for 48 hr at 35 to 37 C under candle extinction. Again make Gram stains and observe the colonies under the microscope [(6), Fig. 1]. Those which fail to meet the above colony description are not likely to be *C. vaginale*.

If the colonies appear typical and uniform, proceed by inoculating one or more PSD slants [(7), Fig. 1] for catalase testing [(8), Fig. 1], maintenance, and to obtain antigen for serological

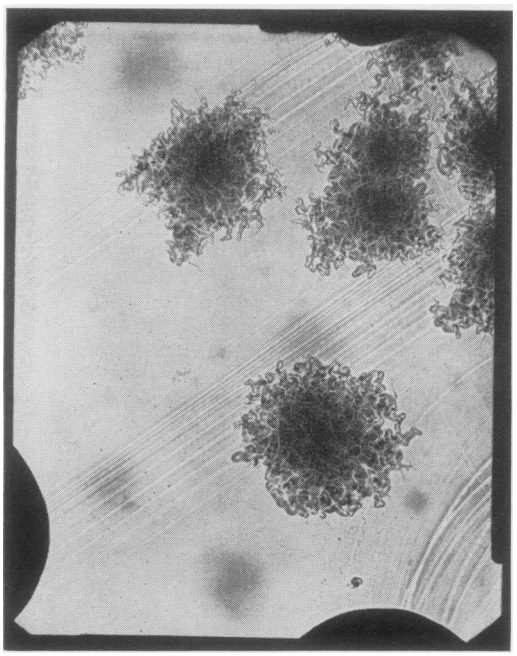

FIG. 5. *Serpentine colonies of lactobacilli on PSD agar after 48 hr of incubation.* ×40.

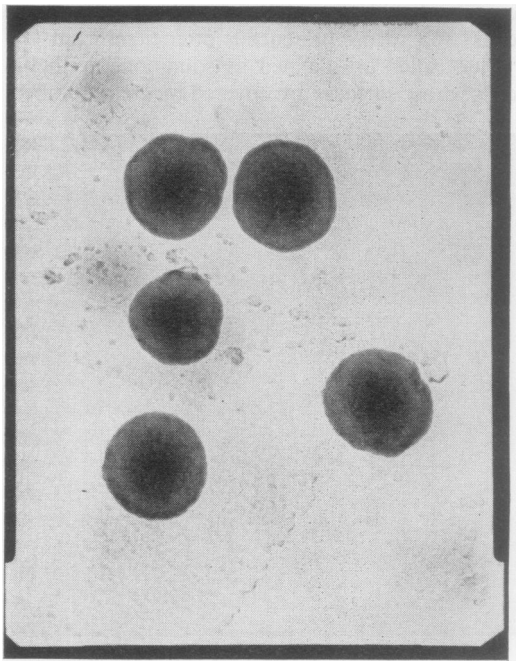

FIG. 6. *Colonies of streptococci on PSD agar after 48 hr of incubation.* ×40.

procedures, if antiserum is available. If the cell mass of the secondary isolates is sufficient, test for H_2O_2 inhibition by inoculating a PSD plate heavily over the entire surface and placing a loopful of 3% H_2O_2 in the center of the plate [(9), Fig. 1]. Do not spread the peroxide. Inoculate the carbohydrate tubes [(10), Fig. 1] by multiple stabs (dextrose, maltose, starch, and PSD with indicator).

Incubate plates and cotton-plugged tubes under increased CO_2 by candle extinction, screw-capped tubes under air with caps on firmly, and all cultures at 35 to 37 C.

Typical *C. vaginale* isolates have the following characteristics. In vaginal exudates, they appear as small, predominantly gram-negative rods or coccobacilli which may show gram-positive beading, and some cells may be frankly gram-positive. The gram negativity tends to increase during subculture on PSD medium, but the irregular staining properties tend to remain.

H_2O_2 overlaid on the PSD slant growth elicits no gas bubbles as *C. vaginale* is catalase-negative. Some unclassified diphtheroids display a delayed positive catalase reaction which means that the test should be observed for not less than 2 min.

Centrally spotted H_2O_2 on a lawn of *C. vaginale* causes a wide zone of growth inhibition (Fig. 8). The test lacks specificity as most catalase-negative

unclassified diphtheroids show the same sensitivity to peroxide, but lack of a zone indicates that the isolate is not *C. vaginale*.

Typical isolates will ferment dextrose weakly to moderately and ferment maltose and starch moderately to strongly in 48 to 72 hr. It is not necessary to incubate the fermentation tubes under increased CO_2, but put the screw caps on firmly.

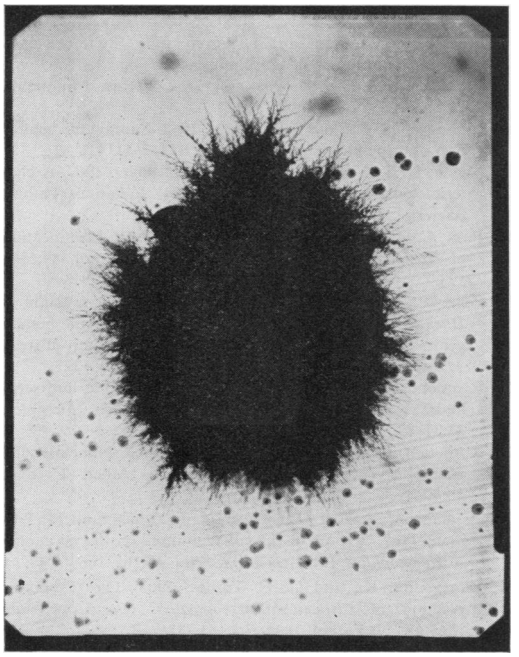

FIG. 7. *Colony of Candida species on PSD agar after 48 hr of incubation.* ×22.

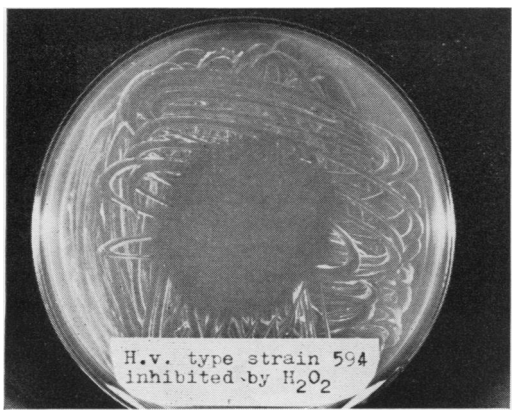

FIG. 8. *C. vaginale (H. vaginalis) on PSD agar after 48 hr of incubation. A loopful of H_2O_2 centrally spotted gave an inhibition zone having a radius of approximately 12 mm.*

DISCUSSION

Identification of *C. vaginale* is based on separation from other *Corynebacterium* species and unclassified coryneform rods common to the genitourinary system. True members of the genus *Haemophilus* do occur in vaginal specimens, but they will not grow on PSD agar.

Most, but not all, vaginal specimens containing *C. vaginale* display characteristic "clue cells" in wet mounts. The microscopic observation in Gram-stained preparations of vaginal exudates is characterized by a profusion of small gram-negative or gram-variable rods associated with epithelial cells.

Concurrent evaluation of the organisms forming the colonies is most important. If an organism is frankly gram-positive on initial isolation and remains so after cultivation on PSD agar, it is not *C. vaginale*. Subcultures of such strains usually reveal that the colonial morphology no longer resembles that of *C. vaginale*.

All *C. vaginale* isolates display a large zone of inhibition on PSD agar plates centrally spotted with 3% H_2O_2. K. Zinnemann of Leeds University suggested this procedure. We do not know at this time whether H_2O_2 is the active agent which allows respiratory streptococci and pneumococci to inhibit *C. vaginale*, but it seems possible (12).

Many of the gram-positive and gram-variable unclassified diphtheroids we have isolated are catalase-negative and remain negative through subculture. This limitation on the importance of the catalase test should be kept in mind. All authors seem to agree, however, that an organism which produces catalase cannot be *C. vaginale*.

Laughton (7) studied 34 vaginal diphtheroids, none of which fermented starch; but some did ferment dextrose and maltose. We have isolated starch-fermenting vaginal diphtheroids, but it appears that all *C. vaginale* isolates are able to ferment starch and most of the unclassified diphtheroids cannot. We have encountered a few strains of *C. vaginale* (National Communicable Disease Center strain A2508 is an example; 2) which show a delayed acid reaction in starch. Use of heavy inocula tends to minimize this problem. Glycogen may be superior to starch as a primary energy source, but cost precludes its routine use.

With the exception of dextrose and dextrose polymers, the taxonomic value of *C. vaginale* reactions on carbon compounds is limited. Other sugars, such as arabinose, xylose, rhamnose, levulose (6), galactose (5), sucrose (9), inulin, and glycerol (17), are attacked weakly or irregularly.

Serological identification has not been stressed because commercially prepared antiserum is not

available. Redmond and Kotcher (14) reported the presence of a specific common antigen in *C. vaginale*, which indicates that sero-identification may be feasible. Their antisera were tested for specificity against various *Haemophilus* species at a time when *C. vaginale* was thought to be a member of genus *Haemophilus*. Cross-reactions between *C. vaginale* and unclassified vaginal diphtheroids have not been investigated fully. Pease and Laughton (13) found a slight cross-reaction between one strain of *C. vaginale* and one strain of *C. cervicis*. It was shown by biochemical and other tests that *C. vaginale* is not *C. cervicis* or *C. parvum* (17).

Park et al. (12) reported that their strains of *C. vaginale* were sensitive to penicillin and resistant to colymycin, nalidixic acid, and neomycin. All of our tested *C. vaginale* isolates conformed to their findings. We do not include this antibiogram in the method, because most unclassified diphtheroids have an identical pattern and there is evidence that some *C. vaginale* strains are resistant to penicillin (6).

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